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PROCESS FOR PURIFYING, STABILIZING, OR ISOLATING NUCLEIC ACIDS FROM BIOLOGICAL MATERIALS

[Verfahren zur Reinigung, Stabilisierung oder Isolierung von Nukleinsäuren aus biologischen Materialien]

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This invention relates to a method of purifying, stabilizing and/or isolating nucleic acids from biological materials, characterized in that an adsorption matrix based on carbohydrates (e.g. potato flour) is added to a sample of biological materials containing nucleic acid for binding impurities.

Description /1

The present invention relates to a method of stabilizing, purifying, and/or isolating nucleic acids from biological materials by removing impurities, e.g., substances that harm nucleic acids and inhibit enzymatic reactions. This method is suitable, in particular, for analyzing, detecting, or isolating nucleic acids in stool samples. Moreover, a method is revealed for implementing a reagent kit suitable for the invention.

Numerous examples from various fields of research confirm the importance of analyzing nucleic acids from biological materials that are contaminated with substances that harm nucleic acids during storage and inhibit enzymatic manipulation of the nucleic acids, e.g., by amplification. Thus, in order for the nucleic acids contained in the biological materials to be useful for further analysis, it is important that these substances be present only at very low concentrations or be completely removed from the sample.

Of particular importance is the analysis of nucleic acids from fecal samples. The main medical application of this is to detect

*Numbers in the margin indicate pagination in the foreign text.

tumor-specific changes in nuclear DNA from fecal matter, which may serve as parameters for the early diagnosis of tumors in the digestive tract. Moreover, detecting bacterial and viral infection pathogens in stool samples using test methods based on nucleic acids is becoming increasingly important.

A method of isolating nucleic acids from stool samples is /2 disclosed in WO 93/20235. However, this method produces low yields of nucleic acids. Moreover, DNA-damaging and/or PCR-inhibiting substances are not separated out. Consequently, the isolated DNA cannot be stored for long periods of time and an amplification to increase specific gene sections of this DNA to be analyzed fails to produce reproducible results. A particularly serious disadvantage of the known method is that PCR amplification produces no intact DNA fragments of uniform sequence, which are needed for further analysis. Obtaining the latter requires expensive cloning of the amplified gene sections. An additional disadvantage of the prior-art method is that the solvents phenol and chloroform must be used, which are extremely harmful to health.

Thus, one object of the present invention was to produce a method by which nucleic acids in biological materials can be stabilized against degradation and in which substances inhibiting the enzymatic manipulation of nucleic-acids can be removed. In particular, a method was to be produced that would make possible reliable isolation of nucleic acids from fecal samples.

This object is achieved by a method of purifying, stabilizing, and/or isolating nucleic acids from biological materials" that is characterized in that an adsorption matrix for binding impurities is added to a nucleic-acid-containing sample of biological materials and then the nucleic acids optionally separated from the bound impurities. The nucleic-acid-containing sample can be brought into contact with the adsorption matrix either directly or after the sample is taken up in a liquid.

Using the method of this invention can significantly improve /3 the usefulness of nucleic acids isolated from biological materials, particularly DNA. Moreover, addition of the adsorption matrix removes large portions of both nucleic-acid-harming substances and materials that inhibit enzymatic manipulation. Thus, the nucleic acids stabilized by the method of the present invention can be stored for long periods of time. Moreover, reproducible results are obtained from amplification of the nucleic acids treated by adding an adsorption matrix, e.g. by PCR,. This reproducibility is essential to the information provided by the results during nucleic-acid analysis. The high quality of nucleic acids purified by the method of this invention is indicated, for example, by the fact that they can be directly examined by sequencing or heteroduplex analysis. Cloning is unnecessary. In addition, the method of this invention requires the use of no harmful solvents.

The adsorption matrix used with the method of this invention is one that can bind impurities that harm nucleic acids and/or prevent enzymatic reactions and/or inhibit enzymatic reactions, such as degradation products of hemoglobin, e.g. bilirubin and its degradation products, and/or bile acids or salts thereof or their degradation products and other degradation products of plant or animal origin. Preferably, an insoluble adsorption matrix is used, since this makes separation from the sample easier.

Good results are obtained using an adsorption matrix based on carbohydrates and/or polypeptides. Preferable is an adsorption matrix based on carbohydrates, e.g., an adsorption matrix containing polysaccharides. Particularly preferable is an adsorption matrix that contains carbohydrates with α- and/or ß- glycosidic linkages, e.g., starch, cellulose, glycogen, and/or other biogenic or nonbiogenic carbohydrates and derivatives or mixtures thereof. /4

Most preferable is the use of flours, i.e., essentially a mixture of cellulose, starch, lipids, and salts, or components thereof. For example, flours of grain, corn, peas, soy, and potatoes or components or mixtures thereof have proven suitable. To those skilled in the art, it is obvious that in addition to the above-mentioned types of flour, other types or mixtures of several types of flour or components thereof can also be used. Most preferred is the use of potato flour or components thereof. Also preferred are mixtures of purified carbohydrates, e.g., cellulose, and flours, such as potato flour.

Also preferred is the use of an adsorption matrix based on carbohydrates, together with soluble components from flours, in particular from one or more of the above-mentioned flour types.

The quantity of adsorption matrix added to the biological sample depends on the type of sample, i.e., the amount of impurities. Good results have been obtained by using the adsorption matrix at a weight proportion of 0.05:1 to 100:1 with reference to the sample containing the nucleic acids. It is particularly preferable to use the adsorption matrix at an amount of 0.1:1 to 10:1.

The nucleic-acid-containing sample to be stabilized by the method of this invention comes from biological materials that contain impurities that degrade nucleic acids or inhibit enzymatic reactions. Preferably, the nucleic-acid-containing sample comes from feces. However, it can also come, for example, from other sources, such as any kind of tissue, bone marrow, human and animal body fluids such /5 as blood, serum, plasma, urine, sperm, cerebrospinal fluid, sputum, and smears, plants, plant parts and extracts, e.g., saps, fungi, microorganisms such as bacteria, fossils or mummified samples, soil samples, clarifier sludge, waste water, and food. The impurities may be, for example, degradation products of hemoglobin, such as bilirubin and its degradation products, and/or bile acids or salts thereof or their degradation products, but also other types of impurities.

For easier handling of the process it has proven favorable to take up the sample of biological materials in a buffer solution before

adding the adsorption matrix. The sample with the adsorption matrix can be incubated at room temperature. The incubation time can be varied over a wide range. After incubation, the adsorption matrix can be separated from the sample, for example, by centrifugation. Alternatively, the adsorption matrix can be added directly to the sample, e.g., in the case of liquid biological samples. Moreover, the sample can be passed over an adsorption matrix by centrifugation, application of a vacuum, and/or by means of gravity, whereby in this case the adsorption matrix is preferably packed in a column.

Treatment with the adsorption matrix leads to a significant increase in stabilization of the nucleic acids contained in the sample and subsequent isolation of the nucleic acids results in better reproducibility. This is true, in particular, if the isolation is followed by enzymatic manipulation of the nucleic acids, e.g., amplification and/or restrictive cleavage. Particularly preferable is amplification, e.g., by PCR (Polymerase Chain Reaction), LCR (Ligase Chain Reaction), NASBA (Nucleic Acid Base Specific Amplification), or 3SR (Self Sustained Sequence Replication).

A particularly preferred aspect of the present invention is /6 the analysis, detection, or isolation of nucleic acids, in particular DNA, from stool samples. Using the method of this invention, pure and amplifiable nucleic acids can be obtained from fecal samples that can be used for detecting mutations, in particular tumor-specific DNA-mutations.

The method of this invention is of considerable importance in tumor diagnosis, since it makes possible the specific detection of nuclear eukaryotic nucleic acids in the presence of impurities and large numbers of bacterial nucleic acids.

Analysis of fecal DNA by applying the method of this invention makes it possible to diagnose tumors of the digestive tract, in particular pancreatic or intestinal tumors, earlier and more accurately. This diagnosis is made, for example, by examining oncogenes and/or tumor-suppressor genes for tumor-specific DNA-mutations. Detection of tumor-specific DNA-mutations in the stool using the method of this invention is possible because cells of tumors in the digestive tracts continuously scale off into the stool. The method of this invention also permits monitoring of therapies aimed at eliminating a tumor and the reliable implementation of tumor-prevention examinations.

Unlike the only non-invasive prior-art routine test for colorectal tumors, the test for occult blood in the stool, the method of this invention seldom if ever results in false-positive results. Moreover, the detection of mutations in genes that mutate in the adenoma stage, i.e., at a very early stage of tumor progression, makes possible a much earlier and more specific diagnosis than was possible with the stool blood test. Suitable objects of the mutation analysis are, in particular, the tumor-suppressor gene APC (Adenomatous Polyposis Coli) (Fearon and Vogelstein (1990), Cell 61,

759-761) and the ras oncogene. Mutation analysis of these two genes in fecal DNA samples can detect, in particular, intestinal tumors, e.g., tumors of the intestines and pancreas. Of course, in addition to the APC gene and the ras gene, additional tumor-relevant genes can also serve as objects of analysis for cancer diagnosis. /7

Apart from tumor-relevant genes, non-translated, repetitive sections of the genome can also serve as objects of analysis in the cancer diagnosis. These so-called microsatellite sections are amplified and the band pattern obtained by gel electrophoresis is compared to band patterns of DNA of healthy body material from the same patient. Different band patterns can point to the presence of a tumor.

An additional example showing the application of the method of this invention is a precise identification of people by examining purified nucleic acids from feces or body materials in forensic analysis. For this purpose, repetitive polymorphous sections of the genome are amplified and the amplification products are separated by gel electrophoresis. The person in question can then be identified by comparing the resulting band pattern with DNA patterns of other suspicious or closely related individuals.

An additional important application of the method of this invention for isolating DNA from fecal samples is found in zoobiological, population-genetic, evolution-genetic, and botanical studies and examinations of animals and plants. In the past, such

examinations have frequently failed because the animal species is rare and the probability of finding the animals in question in a certain locale is low. If the approximate location is known, an analysis of the feces left behind using the method of this invention can provide an important clue as to the degree to which animals are related, which paths they have taken, or what the eating patterns of /8 animals are. Moreover, analysis of fecal nucleic acids, e.g., by detecting microbial or viral nucleic acids, can reveal important diagnostic information on infections, e.g., of a bacterial or viral nature.

An additional object of the present invention is a reagent kit for stabilizing and purifying nucleic acids from biological materials, comprising:

(a) a buffer suitable for taking up a nucleic-acid-containing sample and

(b) an adsorption matrix for binding impurities from the biological materials.

The adsorption matrix can be made available in a packed and portioned form, e.g. filled in a column such as a centrifugable minicolumn.

Preferably, the reagent kit contains additional means of purifying nucleic acids including, for example, mineral and/or organic carriers and optionally solutions, auxiliary materials, and/or accessories. Mineral components of carriers be, for example, porous or

nonporous metal oxides or metal oxide mixtures, et aluminum oxide, titanium dioxide, or zirconium dioxide, silica gels, materials based on glass, e.g., modified or unmodified glass particles or glass powder, quartz, zeolites, or mixtures one or more of the above-mentioned substances. On the other hand, the carrier can also contain organic components that may be chosen, for example, from among the following, optionally with modified functional groups: latex particles, synthetic polymers such as, for example, polyethylene, polypropylene, polyvinylidene fluoride, in particular ultrahigh-molecular polyethylene or HD polyethylene, or mixtures of one or more of the above-mentioned substances. /9

The carrier may be present, for example, in the form of particles having a mean size of 0.1 μm to 1,000 μm. When a porous carrier is used, an average pore size of 2 μm to 1,000 μm is preferred. The carrier may, for example, be used in the form of bulk particles, filter layers, e.g., of glass, quartz, or ceramic, membranes, e.g., membranes in which a silica gel is placed, fibers or fabric of mineral carriers such as quartz or glass wool, or in the form of lattices or fritted materials made of synthetic polymers.

Moreover, the reagent kit can also contain suitable solutions, such as wash solutions or buffer solutions for taking up the sample. A buffer that is suitable for taking up a nucleic-acid-containing sample is, for example, a buffer system based on tris-HCl, pH 8.5-9.5, EDTA, and optionally NaCl. A particularly preferred buffer, especially for

taking up stool samples, contains 500 mM tris-HCl, pH 9, 50 mM EDTA, and 10 mM NaCl.

Moreover, the reagent kit of this invention can also contain auxiliary materials such as enzymes and other agents for manipulating nucleic acids, e.g., at least one amplification primer and enzymes suitable for amplification of nucleic acids, e.g., a nucleic acid polymerase and/or at least one restriction endonuclease.

The primers for amplifying nucleic acids expediently originate from the genes that are to be analyzed, i.e., for example, from oncogenes, tumor suppressor genes, and/or microsatellite sections. Enzymes and restriction endonucleases suitable for amplification of nucleic acids are known and commercially available.

The present invention will be explained with reference to the /10 following example.

Example 1

Analysis of DNA from stool samples

The following adsorption matrices were tested: immobilized bovine serum albumin (BSA), cellulose, and potato starch (all from Sigma, Munich, DE), and potato flour (Honig, Postbus 45, 1540 AA Koog a/d Zaan, NL), which is essentially an insoluble mixture of cellulose, starch, lipids, and salts.

Human stool samples were collected, frozen, and stored at -80°C. 200 mg stool was homogenized in 600 µl stool buffer solution (SBS: 500

mM tris-HCl, pH 9.0, 50 mM EDTA, 10 mM NaCl). 200 µl SBS with 100 mg of the adsorption matrix in question was added, respectively, to 1/4 of the homogenate. The mixture was shaken vigorously and centrifuged twice at 500 g and 13,000 g for 5 min each to precipitate bacteria and other impurities. After treatment of the clear supernatant with proteinase K at a concentration of 2.5 mg/ml, the DNA was purified using a DNA spin column (Qiagen, Hilden, DE), which can be used for purifying DNA from blood and tissue. the column-loading and washing steps were carried out as indicated by the manufacturer.

The DNA was then eluted out of the spin column at a final volume of 150 µl distilled water and stored until use at -20°C. The yield of chromosomal DNA was determined by measuring the absorption at 260 nm.

All the preparations showed comparable total DNA quantities of 15-20 µg. No differences were found between the genomic DNA from preparations with or without the adsorption matrix on an analytical agarose gel. Adding the adsorption matrix also led to no /11 discernable increase in the yield of the extracted chromosomal DNA.

For testing the stability of the isolated nucleic acids, the DNA was examined after being stored for one week. The results are shown in Table 1. The most stable DNA samples were obtained after using potato flour as the adsorption matrix.

3 µl of the purified chromosomal DNA in a total volume of 50 µl was used for amplification by PCR, containing 10 mM tris HCl, pH 8.3,

50 mM KCl, 1.5 mM/$MgCl_2$, 30 µM each of dATP, dCTP, dGTP and dTTP, 400 nM of each primer, 100 µg/ml BSA, and 0.75 units of taq polymerase (AGS, Heidelberg, DE).

To improve sensitivity, nested PCR methods (cf. Jackson, et al., (1991), in McPherson, N.J. Quirke, P. Taylor, G.R. (ed.), PCR-A Practical Approach, Oxford University Press) were implemented, using biotin-labeled nested primers. A PCR of DNA samples purified in the absence of an adsorption matrix was completely blocked. By adding BSA, cellulose, or potato starch as an adsorption matrix, it was possible to obtain partially reproducible PCR results (table 1).

Reproducible PCR results were obtained in all ten analyzed samples when potato flour was used as the adsorption matrix. The PCR fragments were suitable for use in the heteroduplex analysis and also for direct sequencing. For this purpose, single-stranded DNA was prepared using streptavidin-coupled magnetic beads (Dynal, Hamburg, DE), according to the manufacturer's instructions.

TABLE 1: Properties of nuclear DNA from stool /12

Matrix	Loss[a]	PCR[b]
-	80%	0
BSA	60%	3
Cellulose	60%	4
Potato starch	60%	4
Potato flour	< 5%	10

[a] The loss of DNA due to degradation was measured following storage for 1 week at -20°C by analytical agarose gel electrophoresis and spectrophotometric analysis.

[b] A PCR of DNA from 10 different stool samples was carried out. The number of samples analyzable by PCR is indicated.

Example 2

Purification of stool samples

Buffers used:

Buffer SBS:	(see Example 1)
Buffer A:	5.6 M guanidinium/HCl; 20% Tween 20
Buffer B:	10 mM tris/HCl pH 7.5; 100 mM NaCl; 70% ethanol
Buffer C:	10 mM tris/HCl pH 9.0; 0.5 mM EDTA

3 g of frozen stool sample was weighed and mixed with 2 ml buffer SBS by thorough vortexing. The a column was filled with 1:1 mixture (w/w) of potato flour and cellulose and placed in a 50 ml centrifugation tube. The sample taken up in the buffer was transferred to this column and clarified of impurities by /13 centrifugation at 500 rpm for 5 min.

0.125 ml proteinase K stock solution (1.785 mg/ml) and 1.2 ml buffer A were added to 1.2 ml clarified sample. The sample was mixed by vortexing for 1 min and then incubated for 10 min at 70°C. After 1.3 ml absolute alcohol was added and thoroughly mixed, the solution was transferred to a Qia AMP midi column (Qiagen) and the nucleic acids bound to a silica matrix by centrifugation.

The bound nucleic acids were purified by washing them twice with 2.5 ml buffer B and eluted with 0.5 ml buffer C from the Qia AMP midi column and stored at -20°C for later use.

Reproducible results were obtained when a PCR was conducted on

the stored samples as indicated in Example 1.

Claims /14

1. A method of purifying, stabilizing, and/or isolating nucleic acids from biological materials, characterized in that an adsorption matrix for binding impurities is added to a nucleic-acid-containing sample of biological materials.

2. A method as recited in Claim I, characterized in that an adsorption matrix based on carbohydrates is used.

3. A method as recited in Claim 2, characterized in that an adsorption matrix is used that contains carbohydrates with α- and/or ß-glycosidic linkages.

4. A method as recited in one of the Claims 1-3, characterized in that an adsorption matrix is used that contains starch, cellulose, glycogen, and/or other biogenic or nonbiogenic carbohydrates or mixtures thereof.

5. A method as recited in one of the Claims 1-4, characterized in that a flour of grain, peas, corn, soy, potatoes, or components or mixtures thereof is used as the adsorption matrix.

6. A method as recited in Claim 5, characterized in that potato flour or components thereof are used.

7. A method as recited in one of the Claims 1-6, characterized /15 in that an adsorption matrix based on carbohydrates together with soluble flour components.

8. A method as recited in one of the Claims 1-7, characterized in

that mixtures of purified carbohydrates and/or flours are used as the adsorption matrix.

9. A method as recited in one of the Claims 1-8, characterized in that mixtures of cellulose and potato flour are used as the adsorption matrix.

10. A method as recited in one of the Claims 1-9, characterized in that the adsorption matrix is added at a weight ratio of 0.05:1 to 100:1, with reference to the nucleic-acid-containing sample.

11. A method as recited in one of the Claims 1-9, characterized in that the nucleic-acid-containing sample originates from human or animal tissues, bone marrow, body fluids, plants, plant parts and extracts, fungi, microorganisms, fossils or mummified samples, soil samples, clarified sludge, waste water, feces, and foods.

12. A method as recited in one of the Claims 1-11, characterized in that the nucleic-acid-containing sample contains as impurities substances that harm nucleic acids and/or inhibit enzymatic reactions.

13. A method as recited in one of the Claims 1-12, /16
characterized in that the nucleic-acid-containing sample contains as impurities degradation products of hemoglobin and/or bile acids or salts thereof and/or degradation products of plant or animal origin.

14. A method as recited in one of the Claims 1-13, characterized in that the sample of biological materials is taken up in a buffer solution before the adsorption matrix is added.

15. A method as recited in one of the Claims 1-14, characterized in that the adsorption matrix is directly added to the sample of biological materials.

16. A method as recited in one of the Claims 1-15, characterized in that the sample is passed over the adsorption matrix by centrifugation, application of a vacuum, and/or by means of gravity.

17. A method as recited in one of the Claims 1-16, characterized in that isolation of the nucleic acids is followed by direct analysis thereof.

18. A method as recited in one of the Claims 1-17, characterized in that isolation is followed by enzymatic manipulation of the nucleic acids.

19. A method as recited in Claim 18, characterized in that the enzymatic manipulation includes amplification and/or restrictive /17 cleavage.

20. A method as recited in Claim 19, characterized in that the amplification is carried out by PCR (Polymerase Chain Reaction), LCR (Ligase Chain Reaction), NASBA (Nucleic Acid Base Specific Amplification) or 3SR (Self Sustained Sequence Replication).

21. Use of a method as recited in one of the Claims 1-20 for analysis, detection, or isolation of nucleic acids from stool samples.

22. Use as recited in Claim 21 for detecting DNA-mutations.

23. Use as recited in Claim 21 or 22 for analysis, detection, or isolation of nuclear eukaryotic nucleic acids.

24. Use as recited in Claim 23 for diagnosing tumors of the digestive tract, in particular tumors of the pancreas or intestines.

25. Use as recited in Claim 24, characterized in that oncogenes, tumor-suppressor genes, and/or microsatellite sections are examined.

26. Use as recited in Claim 23 for investigating animals and plants.

27. Use as recited in Claim 21 for detecting microbial or viral nucleic acids.

28. Use as recited in Claim 27 for diagnosing bacterial and viral infections. /18

29. Use of a method as recited in one of the Claims 1-20 for demonstrating relations and for forensic identification of individuals.

30. A reagent kit for purifying and stabilizing nucleic acids from biological materials, comprising:

(a) a buffer suitable for taking up a nucleic-acid-containing sample and

(b) an adsorption matrix for binding impurities from the biological materials.

31. A reagent kit as recited in Claim 30, also including agents for further purifying nucleic acids.

32. A reagent kit as recited in Claim 31, characterized in that the agents for additional purification of nucleic acids include

mineral and/or organic carriers and optionally solutions, auxiliary materials, and/or accessories.

33. A reagent kit as recited in Claim 32, characterized in that the carrier contains mineral components of porous or nonporous metal oxides or mixed metal oxides, silica gels, materials based on glass or quartz, zeolites, or mixtures thereof.

34. A reagent kit as recited in Claim 32, characterized in that the carrier contains organic components of optionally modified latex, synthetic polymers, or mixtures thereof.

35. A reagent kit as recited in one of the Claims 32-34, /19 characterized in that the carrier is present in the form of particles with a mean size of 0.1 μm to 1,000 μm.

36. A reagent kit as recited in one of the Claims 32-35, characterized in that the carrier has pores having a mean size of 2 μm to 1,000 μm.

37. A reagent kit as recited in one of the Claims 32-36, characterized in that the carrier is present in the form of bulk particles, filter layers, membranes, fabrics, fibers, or fritted materials.

38. A reagent kit as recited in one of the Claims 30-37, characterized in that the adsorption matrix is filled into a column.